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Bioavailability of caseinophosphopeptide bound iron in the young rat

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Iron forms strong and soluble complexes with 1–25 caseinophosphopeptide (CPP) issued from the enzymatic hydrolysis of β-casein. That could prevent iron from insolubilization and low digestive absorption. Young iron deficient rats (5 mg iron/kg diet for 4 weeks) were repleted (200 mg iron/kg diet, 2 weeks) using either $FeSO_4$ or iron bound to CPP of whole hydrolyzed β-casein (β-cas hydr group) or purified molecule (β-cas (1–25) group). Two other groups were fed a control diet (200 mg/kg as $FeSO_4$) for the 6 weeks, either ad libitum (control) or pair fed (PF) to experimental groups. A metabolic balance was performed during the 2nd week of repletion. Experimental groups displayed a higher iron absorption than control groups, but did not differ among themselves. At the end of the 2 weeks repletion period, $FeSO_4$ and β-cas hydr groups showed similar values of Hb, Hct, and RBC count, which were lower than control and PF groups. Hct and Hb values of the β-cas (1–25) group were higher than β-cas hydr and $FeSO_4$ groups, but did not differ from control and PF animals. MCV values of control and PF groups did not differ from $FeSO_4$ group but were lower than the β-cas (1–25) and β-cas hydr groups. The Fe liver content was significantly higher in peptide-bound Fe groups ((1–25) β-cas and β-cas hydr) than the three other groups; the $FeSO_4$ group showed the lowest levels. Binding iron to phosphocaseinophosphopeptide seems to improve its bioavailability and to hasten the cure of iron deficiency in the young animal.
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Introduction

Iron (Fe) deficiency is the most common nutritional deficiency in both developing and industrialized countries.[1] Infants and young children are at especially high risk because of high requirements corresponding to basal losses, expansion of red cell mass, and rapid growth of body tissues.[2] Low Fe bioavailability prevents efficient treatment of Fe deficiency. The high content of iron-fortified formulas is liable to prevent iron deficiency; however, this source of Fe, such as cow milk has a low absorption rate as compared with breast milk or meat Fe.[3–5] More, these high levels are liable to inhibit the absorption of other essential trace elements such as zinc.[6] Iron absorption depends largely on the effect of dietary factors that enhance or inhibit its absorption: vitamin C or the presence of Fe as hemoglobin increase Fe absorption, whereas calcium and phytates decrease it.[4,5,7,8] Proteins can both enhance or impair Fe absorption, depending on their source: meat proteins enhance Fe absorption;[9] egg white, cow milk proteins, and even more soy proteins display an inhibiting effect;[4,7,9,10] the effect of cow milk proteins is lessened by their enzymatic hydrolysis, which enhances their digestive solubility;[10,11] solubility of Fe increases as protein digestion progresses.[12] Fe absorption is correlated with its intestinal solubility.[13] In addition, milk proteins present interesting binding properties for divalent cations that could be used, together with their hydrolysis, to improve Fe bioavailability.[14,15] One third of the casein content of bovine milk is made of β-casein, which can bind seven divalent cation atoms at pH 7.0. Among its 209 amino acid residues, it

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displays five phosphoserines (Ser (P)) located at positions 15, 17, 18, 19, and 35. Tryptic hydrolysis of β-casein leads to the production of a phosphopeptide (1–25) containing four phosphoseryl residues: this caseinophosphopeptide (CPP) is partially resistant to further hydrolysis by pancreatic enzymes;[16–18] the occurrence of such a phosphopeptide has been reported in the distal small intestine of rats during the course of the luminal digestion of a diet containing caseins;[18,19] CPP is able to form soluble complexes with di- or trivalent ions.[15,16,20–22]

The main effect of Ca binding to CPP is to improve its solubility, even if the resulting effect on digestive absorption is still debated,[19,23,24] but the influence of CPP on the metabolism of other elements has never been tested. Fe binding to phosphoseryl residues is very strong, about 100 times over Ca binding,[15,21] and seems to resist pH variations that occur during gastric and upper duodenal transit;[22] these changes usually dissociate Fe from ligands and lead to its polymerization and its insolubilization.[3,4] Fe binding ability is not altered by ionic strength because of the presence of coordination links.[20,21] Therefore, binding Fe to phosphopeptides issued from the hydrolysis of β-casein could keep it soluble during upper digestive tract transit, prevent interactions with other minerals or trace elements, and favor its digestive absorption.

However absorption is only the first step of Fe metabolism. A high bioavailability implies that the absorbed Fe is efficiently used for erythropoiesis or is stored in a form that allows mobilization for that purpose, i.e., liver ferritin.[3] This work was designed to study the effect of binding Fe to caseinophosphopeptide issued from β-casein hydrolysis on its bioavailability in the young Fe-deficient rat, using metabolic balance and red blood cell (RBC) counts, and the direct measurement of Fe storage in liver.

Methods and materials

Animals, housing, and diets

Weanling male Sprague Dawley rats (issued from the farm of the University of Caen France), 23 days old, weighing 50 to 60 g, were housed individually in plastic and stainless steel wire bottom metabolic cages. In the first step, the animals were divided into four groups of eight rats. The control group (Control) was fed a standard diet (20% protein as casein; 200 mg Fe/kg diet as $Fe++SO_4$) for 6 weeks. The three other groups were fed an iron-deficient diet (<5 mg Fe/kg) (UAR, Villemoisson-sur-Orge France) for 4 weeks. The composition of the Fe-deficient diet was otherwise similar to the control diet. The detailed composition of the diet is given in the *Table 1*. The deficient and control rats had free access to diet and distilled water. A fifth group (PF) was pair-fed to the Fe-deficient groups with the normal iron-containing diet.

After the first 4 weeks, the three experimental groups were fed the same iron deficient diet to which Fe had been added to a level of 200 mg/kg. Fe sources were $Fe++SO_4$ (group $FeSO_4$), Fe bound to hydrolyzed β-casein (group β-cas hydr) (2% diet), and Fe bound to (1–25) CPP, group β-cas (1–25). Iron was added in a dried form. During these 2 weeks the five groups were fed diets and distilled water ad libitum; diets had a similar content in Fe (200 mg/kg) and protein (20%). Animals were weighed on arrival in the laboratory and weekly thereafter.

Table 1 Composition of the diet*

Nutrients	
Protein	20%
Lipids	4.4%
Carbohydrates	56.4%
Cellulose	4.3%
Minerals and Vitamins	5.5%
Iron	5–200 mg/kg
Moisture	9.7%

*Diet deprived of protein (UAR Aprotéique) to whom casein or casein+β-casein derived peptides were added to a final level of 20%. Components of UAR Aprotéique: protein: 0%; glucose + starch: 80%; cellulose: 6%; lipids: 6%; minerals: P (7.75 g/kg), Ca (10 g/kg), K (6 g/kg), Na (4 g/kg), Mg (1 g/kg), Mn (80 mg/kg), Zn (45 mg/kg), Cu (12,5 mg/kg); vitamins: A (19800 UI/kg), D (2500 UI/kg), B1 (20 mg/kg), B2 (15 mg/kg), B6 (10 mg/kg), E (170 mg/kg), K (40 mg/kg), PP (100 mg/kg), Biotin (0.3 mg/kg), Folic Acid (5 mg/kg).

Hematological tests

Blood was drawn for a blood cell count at the end of the 4th week, and at the end of the experiment. Approximately 100 μL of free-flowing blood from orbital puncture were used for determination of hemoglobin (Hb), hematocrit (Hct), RBC, and mean corpuscular volume (MCV) on a Coulter Counter S890 (Coulter Electronics, Ltd.).

Iron balance and dosages

A 3 day metabolic balance was made in the metabolic cages, 7 days after the beginning of the second period in control and experimental groups. Urines and feces were collected daily, weighed, and frozen until analysis.

Fe absorption is the difference between intakes (diet + water) and excretion (feces + urines). Because of the low urine Fe excretion, the absorption rate is close to retention values.

At the end of the experiment the animals (65 days old) were killed by an overdose of Pentobarbital; liver was excised, weighed, and frozen until analysis. After thawing, feces and organs were dried at 90°C, ground and mixed; feces, organs, and samples of diet were digested with pure nitric acid in a microwave oven (Microdigest A301, PROLABO, France). Urine and tap water were analyzed as such. Samples were analyzed for Fe by Atomic Absorption Spectrometry (Perkin Elmer 1100). Bovine liver standard (National Bureau of Standards, Washington, DC; Standard Reference Material NIST 1577B) was analyzed for Fe concentrations to evaluate our methodological accuracy. Certified concentration was (mean ± 1SD) 184 ± 15 μg/g; laboratory value was (n = 13) 190 ± 6. The run to run coefficient of variation was 1.29% (n = 30).

Purification of β-casein

The β-casein was isolated from industrially made sodium caseinate (Armor Proteines, St-Brice-en Coglés France) using its solubility at pH 4.5 at 4°C followed by ion exchange chromatography.[20] The (1–25) phosphopeptide was prepared using a tryptic hydrolysis of β-casein.[25] Hydrolysate and purified (1–25) phosphopeptide present as a white powder.

Binding of Fe to casein hydrolysate or peptides

β-casein (1–25) or hydrolyzed β-casein at a concentration of 12.5 mg/mL (7.10^{-6} mol) were incubated in a $FeCl_2$ solution (7.10^{-5} mol, pH 5.3) during 30 min at 25°C (Milli Q system, Millipore).

Table 2 Iron balance in rats with iron-deficiency-induced anaemia (<5 mg/kg diet) for 4 weeks and subsequently fed on Fe-sufficient (200 mg/kg), differing in iron-bound content for 2 weeks

Group	Absorption (mg/d)	Absorption (%)	Urine Fe (μg/d)
Controls	1.1 (0.3)	31.5 (7.3)	3.0 (0.5)
Pair fed	1.2 (0.1)	26.6 (3.1)	2.4 (1.0)
FeSO4	1.9 (0.3)[a,b]	49.0 (7.5)[a,b]	1.9 (0.3)
β-Cas hydr	2.2 (0.24)[a,b]	49.0 (3.1)[a,b]	6.1 (1.0)[a,b,c]
β-Cas (1–25)	1.9 (0.1)[a,b]	46.6 (2.5)[a,b]	10.7 (2.0)[a,b,c,d]
	$P = 0.0001$	$P = 0.004$	$P = 0.0001$

All values are means (SE), $n = 8$.
Controls: Fe + whole β casein.
Pair fed: Fe + whole β casein, pair fed to experimental groups.
FeSO4: Fe + whole β casein (4 weeks Fe deficient and 2 weeks Fe sufficient).
β-Cas hydr: Fe bound to hydrolysed β casein.
β-Cas (1–25): Fe bound to the (1–25) caseinophosphopeptide of β casein.
[a]versus controls, [b]versus pair-fed, [c]versus FeSO4, [d]versus β-Cas hydr, ($P < 0.05$, ANOVA and Fisher's test).

The preparation was dialyzed during 24 hr at 4°C, using dialysis bags with a cut-off of 1000 Da. The complexed iron was determined by atomic absorption spectrometry on a Varian spectrometer (Model AA 1275). A dialysis control without β-casein (1–25) was incubated and dialysed with the same conditions.

Statistics

Comparisons between groups were made by two-way ANOVA and Fisher's exact tests on "statView SE + Graphics™" (Abacus Concept, Inc., CA USA).

Results

Neither iron intakes (mg) nor growth (g) differed between the 5 groups during the 2 weeks repletion period: control group (mean ± SE) (55.2 ± mg; 94.2 ± 14.3 g); pair fed (PF) group (64.6 ± 33.4 mg; 97.9 ± 13.2 g); $FeSO_4$ group (51.9 ± 4.6 mg; 106.4 ± 9.9 g); β-cas hydr (67.4 ± 4.8 mg; 105.9 ± 12.0 g); β-cas (1–25) (62.1 ± 6.2 mg; 92.6 ± 12.0 g).

Results of the metabolic balance are displayed in *Table 2*. Fe absorption and retention of experimental groups ($FeSO_4$, β-cas hydr and β-cas (1–25)) were significantly higher than control and PF groups, but did not differ between themselves. However, Fe urine excretion of the two CPP bound Fe groups was higher than in the three $FeSO_4$ groups. The β-cas (1–25) group had higher urine excretion than β-cas hydr group.

Results of blood parameters are given in the *Table 3*. At the end of the deficient period (week 4), the three experimental groups displayed similar values of RBC count, Hb, Hct, and MCV, which were significantly different from control and PF groups. The experimental groups differed only in a higher Hb level of β-cas (1–25) group. At the end of the 2-week repletion period, $FeSO_4$, and β-cas hydr groups showed similar values of Hb, Hct, and RBC count, which were lower than control and PF groups. On the other hand, Hct and Hb values of the β-cas (1–25) group were higher than β-cas hydr and $FeSO_4$ groups but did not differ from control and PF animals. MCV values of control and PF groups did not differ from $FeSO_4$, but were lower than the β-cas (1–25) and β-cas hydr groups.

The Fe liver content is shown in *Table 4*. It was significantly higher in peptide bound Fe groups (β-cas (1–25) and β-cas hydr) than in the three other groups; $FeSO_4$ group showed the lowest levels.

Discussion

Prevention and correction of iron (Fe) deficiency is a hard task because of the poor absorption of the usual Fe salts. More, this absorption is quite variable and subject to nutrient-nutrient interactions in the digestive tract where Fe shows a high susceptibility to polymerization and insolubilization.[3,4] Because of the nutritional value of cow's milk, it should be useful to improve the bioavailability of its Fe, which is considered to be low, mainly because of Ca concentration and protein composition.[4,5,7–9] Although it

Table 3 Iron status parameters of rats with iron-deficiency-induced anaemia (<5 mg/kg diet) for 4 weeks and subsequently fed on Fe-sufficient (200 mg/kg), differing in iron-bound content for 2 weeks

Group	Time point (week)	RBC (10^6/mm^3)	H_b (g/dl)	Hc_t (%)	MCV (μ^3)
Controls	4	6.6 (0.2)	14.2 (0.3)	39.9 (1.1)	56.4 (4.0)
	6[1]	7.0 (0.1)	15.3 (0.3)	41.2 (0.8)	57.1 (2.1)
Pair fed	4	7.1 (0.2)	15.5 (0.3)[a]	42.8 (1.4)[a]	60.5 (0.5)
	6	7.4 (0.2)[a]	15.1 (0.3)	41.0 (1.1)	59.3 (0.3)
FeSO4	4	3.5 (0.2)[a,b]	5.5 (0.4)[a,b]	14.2 (1.0)[a,b]	41.4 (0.3)[a,b]
	6	6.2 (0.1)[a,b]	13.2 (0.3)[a,b]	37.5 (0.8)[a,b]	59.6 (0.8)
β-Cas hydr	4	3.5 (0.1)[a,b]	5.2 (0.2)[a,b]	14.8 (0.7)[a,b]	42.6 (0.7)[a,b]
	6	5.9 (0.1)[a,b]	13.5 (0.2)[a,b]	38.7 (0.8)[a,b]	65.4 (0.7)[a,b,c]
β-Cas (1–25)	4	3.8 (0.1)[a,b]	6.3 (0.3)[a,b,d]	16.6 (0.7)[a,b]	44.1 (0.4)[a,b]
	6	6.5 (0.0)[a,b,d]	15.1 (0.1)[c,d]	41.7 (0.4)[c,d]	64.2 (0.3)[a,b,c]
		$P = 0.0001$	$P = 0.0001$	$P = 0.0024$	$P = 0.0001$

[1]End of the experiment.
All values are means (SE) and $n = 8$.
[a]versus controls, [b]versus pair-fed, [c]versus FeSO4, [d]versus β-Cas hydr, ($P < 0.05$, ANOVA and Fisher's test), at the same period.
[§]For details of diets and procedures, see *Table 2* and text.

Table 4 Liver iron concentrations in rats with iron-deficiency-induced anaemia (<5 mg/kg diet) for 4 weeks and subsequently fed on Fe-sufficient (200 mg/kg), differing in iron-bound content for 2 weeks

Group	Fe liver concentration (mg/g wet wt)	Fe liver content (mg)
Controls	126 (7)	1.8 (0.1)
Pair fed	159 (11)	2.0 (01)
FeSO4	93 (10)[b]	1.3 (0.2)[b]
β-Cas hydr	183 (16)[a,c]	2.7 (0.2)[a,b,c]
β-Cas (1–25)	202 (27)[a,c]	2.5 (0.2)[a,b,c]
	$P = 0.0002$	$P = 0.0002$

All values are means (SE) and $n = 8$.
[a]versus controls, [b]versus pair-fed, [c]versus FeSO4, [d]versus β-Cas hydr, ($P < 0.05$, ANOVA and Fisher's test).
[§]For details of diets and procedures, see *Table 2* and text.

has been shown that the presence of intact casein can impair Fe absorption, this effect can be reduced by hydrolyzing cow's milk proteins.[10,11] In addition, binding Fe to soluble peptides issued from hydrolyzed cow's milk proteins could protect it from interactions with other nutrients. Trypsic hydrolysis of casein yields a number of peptides, some of them display functional, not only nutritional, properties.[26] Among them, the 1–25 caseinophosphopeptide (CPP) includes four of the five phosphoseryl residues of the whole molecule.[5,17,20] It supports the major part of the binding property of β-casein for divalent cations.[17] Fe binding to phosphorylated casein is resistant to dissociation, which occurs at the low gastric pH.[23] Furthermore, CPP is resistant to further enzymatic hydrolysis.[17–19] Therefore, Fe can be kept in a protected, stable state until it reaches the enterocyte receptor.

As expected, the metabolic balance performed during the second week of repletion showed that previously Fe deficient groups had a higher absorption rate than groups fed a control Fe diet, either ad libitum, or pair fed to experimental groups. There was no difference, however, between these experimental groups which provided Fe as a free salt ($FeSO_4$) or complexed to the CPP of β-casein. However the bioavailability of CPP-bound Fe seems to be better than FeS_4, because the group fed Fe bound to the purified (1–25) CPP showed a better improvement of most of the blood parameters of Fe status as compared to $FeSO_4$; in addition both CPP groups (β-cas hydr and β-cas (1–25)) showed higher liver storage than $FeSO_4$ groups, either fed Fe sufficient (control, PF) or deficient diets. The only difference between the two CPP groups was the greater degree of purity and concentration of CPP in the 1–25 CPP than in the hydrolyzed casein that contains in addition nonphosphorylated peptides.

At the end of the repletion period, the improvement of liver storage was more complete in the CPP bound Fe groups (1–25 and hydrolyzed β-cas) than in the $FeSO_4$ group. Therefore, supplying Fe as a CPP bound complex allowed a better improvement in erythropoiesis and Fe storage of Fe deprived young rats than $FeSO_4$. A better storage is also suggested by the greater urine Fe excretion of the two groups CPP groups, compared with $FeSO_4$-fed animals.

The biphasic response of Fe intestinal absorption to deficiency could explain the apparent discrepancy between tissue storage and the results of the metabolic balance. It is known that a high Fe absorption rate is observed as long as anemia is observed; then a sharp decline occurs and Fe stores are reconstituted at a slow rate, suggesting that there are two different regulators of Fe absorption: an erythroid regulator and a store regulator, with the erythroid regulator having the greater capacity.[3] In the CPP groups a better initial absorption efficiency could have allowed an earlier improvement of Hb levels; as a result an early decrease of absorption rate could have occurred, together with a shift of absorbed Fe to the correction of tissue storage. During the second week of repletion therefore, the absorption of Fe could be similar in the three experimental groups while the liver content was yet high in CPP fed groups.

Binding Fe to CPP could improve its bioavailability by several mechanisms: from previous work,[10,11] it can be assumed than protein hydrolysis is liable to enhance Fe absorption, maybe by improving its solubility that is known to be correlated with its absorption.[13] Binding Fe to CPP yields soluble complexes, resistant to changes in pH and ionic strength which occur in the upper digestive tract.[20–22] Therefore, a fraction of CPP escapes complete digestion and is found in feces,[18,19] allowing a longer contact of soluble Fe with the mucosa. Large, nonphosphorylated peptides present in the whole β-casein hydrolysate could have impaired Fe solubility,[12,27] leading to the less obvious effects observed in the β-casein hydr fed group than in the group fed iron bound to purified β-casein (1–25).

On the other hand Fe could also be absorbed linked to the CPP, since intestinal uptake by transcytosis of casein derived peptides has been reported;[26,28] the most important part of these absorbed peptides is broken down inside the enterocyte. Thereafter, the Fe could be submitted to the enterocyte regulation of the absorption.

Conclusions

Binding Fe to soluble peptides issued β-casein hydrolysis appears to improve its bioavailability during repletion of Fe deficiency, in the young rat. Further studies are to be performed to precisely define the mechanisms involved, and to confirm these findings in humans.

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